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Immunohistochemical detection of Survivin in Canine Lymphoma

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Für Ma und Pa

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Immunohistochemical Detection of Survivin in Canine Lymphoma

Summary

Survivin is a member of the family of proteins known as 'inhibitors of apoptosis proteins'. Survivin has a role in cellular decisions concerning division and survival and is frequently expressed in neoplastic cells. The aim of the present study was to investigate immunohistochemically the expression of survivin in normal canine tissues and in canine lymphoma. A representative range of fetal and adult normal tissues as well as biopsy samples from dogs with lymphoma were assembled in tissue arrays. The lymphomas were classified according to the revised Kiel and to the revised European American Lymphoma – World Health Organization (REAL-WHO) schemes. Polyclonal and monoclonal antisera cross-reactive with canine survivin identified cytoplasmic expression of the molecule in a broad range of normal canine cells. The same reagents demonstrated cytoplasmic labelling of more than 5% of cells in all 83 lymphoma samples tested with polyclonal antiserum and in 67 of 82 (82%) of samples tested with monoclonal antiserum. Survivin was expressed by a wide range of canine lymphoma subtypes, but the expression of this molecule in normal canine tissues must be considered if novel therapies targeting survivin are applied to the management of canine lymphoma.

Introduction

Apoptosis is a phylogenetically conserved mechanism of cell death with roles in development and tissue homeostasis (Salvesen and Duckett, 2002). In mammals, it is executed through activation of different caspases, which are present in the cell cytoplasm as inactive proenzymes. Activation of these molecules can be triggered either by external stimuli, which activate the extrinsic apoptotic pathway, or by diverse conditions collectively termed 'cellular stress', which activate the intrinsic pathway. Following stress stimuli, cytochrome c is released from the mitochondrial intermembrane space into the cell cytoplasm, where it participates in the assembly of the apoptosome and subsequent activation of the initiator caspase-9. This enzyme cleaves and activates downstream caspases that target further cellular substrates for cleavage, eventually resulting in cell death (Gerl and Vaux, 2005).

Inhibitors of apoptosis proteins (IAPs) are a family of proteins that interfere with the activation of caspases by various mechanisms (Salvesen and Duckett, 2002). All members of the family possess at least one 'baculovirus inhibitor of apoptosis repeat', a specific domain that, at least in some of its versions, has been shown to bind and/or inactivate caspases. Increased expression of various IAPs has been reported in several types of cancer and is thought to contribute to the phenomenon of resistance to apoptosis that is often a feature of the neoplastic process. Survivin is a member of the IAP family with well known functions in the regulation of cell division. In contrast, its anti-apoptotic function has long been debated and, only recently, relevant mechanisms have been unveiled in tumour cells (Altieri, 2008). In order to inhibit apoptosis, survivin must localize to the cytosol and its negative regulatory effect on caspase function appears to be indirect (Stauber *et al.*, 2007; Altieri, 2008). Overexpression of survivin has been

reported in a wide range of human cancers, and, similarly to other IAPs, it has been shown to negatively influence outcome in many different types of cancer (Stauber *et al.*, 2007). Based mostly on early data from man, it is frequently claimed that survivin is widely expressed during embryonic development, but is not expressed or only weakly expressed in normal adult tissues (Ambrosini *et al.*, 1997; Andersen *et al.*, 2007; Altieri, 2008). Some authors concede that survivin is expressed to some extent in adult tissues with a high cell turnover including testis, intestinal epithelium and bone marrow (Kobayashi *et al.*, 1999). Evidence that the role of survivin is not restricted to developing tissues is emerging, with several reports of its expression in normal adult tissues (Fukuda and Pelus, 2006; Lechler *et al.*, 2007).

Canine survivin is 91.5% homologous to its human counterpart at the amino acid level (Uchide *et al.*, 2005). Immunohistochemical studies have described expression of this protein in canine mast cell tumours (Scase *et al.*, 2006), haemangiomas and haemangiosarcomas (Murakami *et al.*, 2008), transitional cell carcinomas of the urinary bladder (Velando Rankin *et al.*, 2008) and lymphomas (Rebhun *et al.*, 2008). The prognostic significance of this expression remains undetermined. It has been suggested that survivin might be expressed by all canine lymphomas and a negative prognostic significance has been attributed to high expression of survivin in B-cell neoplasms (Rebhun *et al.*, 2008). Survivin mRNA has been detected by reverse transcriptase polymerase chain reaction (RT-PCR) in the tissue of normal adult dogs including heart, lung, liver, stomach, duodenum, colon, spleen, kidney and testis, as well as in various tumour tissues (Uchide *et al.*, 2005). Immunohistochemical studies to confirm expression of survivin protein have not been performed.

The aim of the present study was to establish a reliable method for immunohistochemical detection of survivin and to apply this methodology to a detailed analysis of expression of survivin protein in normal canine tissues and samples of canine lymphoma.

Materials and Methods

Primary Antibodies for Immunohistochemistry

Two commercially available antibodies raised against full-length human survivin were compared in immunohistochemistry (IHC). The first was a polyclonal, affinity purified antibody (survivin-astrocyte marker NB 500-201, Lot Q2; Novus Biologicals Inc., Littleton, Colorado) hereafter described as pSurv in this study. The second was a monoclonal antibody (survivin D8: sc-17779, StC Biotechnology Inc., Santa Cruz, California), referred to as mSurv in this study. The specific epitope recognized by the monoclonal antibody is unknown. Lymphomas were immunophenotyped by application of polyclonal anti-human pan T-cell (CD3) antibody (Dako Cytomation, Zug, Switzerland) and a monoclonal anti-human pan B-cell (CD79a) antibody (Dako Cytomation).

Cloning and Expression of Canine Survivin

Isolation of total RNA from Madin Darby Canine Kidney (MDCK) cells was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols and as described previously (Keller *et al.*, 2007b). Briefly, 40U Protector RNAse Inhibitor (Roche, Mannheim, Germany) were added to the extract. cDNA was synthesized from 1 μg of total RNA using the 1st strand cDNA Synthesis Kit for RT-PCR

(AMV) and an oligo-dT primer according to the manufacturer's instructions (Roche). Amplification of the survivin open reading frame (ORF) was performed by PCR with primers survivinExUp (5'-ATGGGCGCTCGTCGCTG-3') and survivinExDn (5'-GAGCTATTCTGCGGCGGC-3') using Platinum Taq DNA Polymerase High Fidelity™ (Invitrogen, Carlsbad, California). PCR products were cloned into the glutathione S-transferase (GST) pGEX-4T2 bacterial expression vector (GE Healthcare GmbH, Otelfingen, Switzerland) by sticky-end ligation. After transformation of the vector into *Escherichia coli* DH5α competent cells (Invitrogen AG, Basel, Switzerland), individual clones containing an insert of the expected size were sent for sequencing (Microsynth AG, Balgach, Switzerland). The consensus sequence was derived from at least three different clones. A clone containing the consensus survivin sequence was transformed into *E. coli* BL21Star competent cells for protein expression. For expression of GST-survivin and a GST protein only control, transformed cells containing either the survivin expression construct or empty vector were grown at 30 °C in 500 ml of lysogeny broth medium (LB) with 75 µg/ml ampicillin. At an optical density of 0.6 at 600 nm, GST fusion protein expression was induced by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma Aldrich Co., St. Louis, Missouri) with incubation under the same conditions for further 2.5 h. Thereafter the bacterial culture was processed for paraffin wax embedding, western blotting or antibody preincubation experiments.

Western Blotting

Following protein induction, bacterial cultures (1 ml) were centrifuged and the pellets were resuspended in 200 µl of 2x sodium dodecyl sulphate (SDS) buffer containing 10%

β-mercaptoethanol. Samples were incubated at 95°C for 10 min, centrifuged for 5 min and 2 μl of bacterial lysates were loaded onto 12% SDS polyacrylamide gels and separated by gel electrophoresis (SDS-PAGE). After transferring the proteins to a nitrocellulose membrane by means of a semi-dry blotting system (Biometra, Goettingen, Germany), non-specific antibody binding sites were blocked by incubation of the membrane for 1h at room temperature in Tris-buffered saline containing Tween 20 0.05% (TBST) and bovine serum albumin (BSA) 3%. The membrane was washed three times for 15 sec and twice for 10 min in TBST and then incubated for 1 h at room temperature with pSurv (1 μg/ml in BSA 1 %) or mSurv (2 μg/ml in BSA 1%). Following this initial incubation, the membranes were washed again with TBST as described above and incubated with secondary anti-mouse IgG conjugated to horseradish peroxidase (HRP; Geno Technologies, St Louis, Missouri) or anti-rabbit IgG conjugated to HRP (Jackson, ImmunoResearch, Soham, UK) diluted 1 in 10,000 for 1 h at room temperature in BSA 1%. As a control for this analysis, the presence of GST-tagged proteins was detected using mouse anti-GST antibody (0.1 μg/ml) conjugated to HRP (Amersham Biosciences, Otelfingen, Switzerland). Signals were visualized following incubation with a LumiGlo™ substrate (KPL, Maryland) and autoradiography.

Keratinocyte Cultures

Methods for culture, fixation and embedding of normal and neoplastic canine keratinocytes have been described previously (Keller *et al.*, 2007b). Cells used included a normal canine keratinocyte line and a squamous cell carcinoma line (Crameri *et al.*, 1997; Keller *et al.*, 2007b).

Fixation and Embedding of Bacteria Expressing Recombinant Canine Protein

100 ml cultures of *E. coli* BL21 cells, expressing GST-fusion proteins of survivin, XIAP (GenBank Accession number: DQ225116), cIAP1 (DQ223014), cIAP2 (EF102104), Smac/DIABLO (EF088670), Omi/HtrA2 (DQ138643) and GST alone, in addition to control *E. coli* MG1655 wild-type cells were centrifuged at 4,000 g for 10 min at 4°C and the pellets were resuspended in 4% neutral buffered formaldehyd and incubated for 24 h at room temperature. The cultures were subsequently centrifuged at 4,000 g for 10 min at room temperature. The resulting pellets were resuspended in 1.5 ml of 5% BSA in PBS plus 2 drops of hemalum in an Eppendorf tube before a final centrifugation at 4,000 g at room temperature for 10 min. The pellets were embedded in paraffin wax with the Cytoblock system (Thermo Shandon, Pittsburgh, Pennsylvania).

Tissue Array Construction

Tissue arrays were constructed from archival samples by means of a manual tissue array production apparatus (Beecher Instruments, Sun Prairie, USA). To minimize variation, all normal tissue and lymphoma samples used to assemble arrays were derived from patients that had been admitted to the Faculty clinic and all tissues had been fixed in house. The majority of the specimens derived from biopsies. The first array was composed of a broad spectrum of normal fetal and adult canine tissues (free of microscopical lesions) that had been fixed in 4% buffered formaldehyd for 24 to 48 h and embedded in paraffin wax. Fetal tissues included placenta, kidney, liver, pancreas, lung, intestine, skeletal muscle, heart muscle and brain. Adult tissues included placenta, kidney, liver, pancreas, spleen, lung, intestine, heart muscle, skeletal muscle, brain, testis and lymph node. The array was constructed by collecting two cores of 1.2 mm

diameter (fetal tissues) or 0.6 mm diameter (adult tissues) from representative areas of each specimen that were transferred to paraffin wax recipient blocks (Micro-Cut Paraffin, Polysciences Inc., Warrington, USA). Samples for each organ originated from two to three different individuals. A further series of arrays was constructed using 95 samples of canine lymphoma (Keller *et al.*, 2007a). Four cores of 0.6 mm diameter were collected from representative areas of each specimen. Before sectioning, the blocks were immersed in water, kept at -20°C for 15 min, cut into sections of 1.5 μm and transferred to positively charged glass slides (Erie Scientific Company, Portsmouth, New Hampshire).

Survivin Immunohistochemistry

An optimized immunohistochemical procedure for detection of canine survivin was developed by testing a series of different antigen retrieval methods, primary antibody dilutions and incubation conditions (Keller *et al.*, 2007b). The optimized conditions are described forthwith. Antigen retrieval was carried out for both anti-survivin antibodies by boiling the slides in Tris/ethylene diaminetetraacetic acid (EDTA) buffer pH 9 at 98°C for 20 min in a pressure cooker ('Pascal'; Dako Cytomation). The sections were cooled for 10 min and then incubated with either pSurv (1 in 600 dilution or 1.6 μg/ml; 4^{o}C overnight) or mSurv (1 in 10 dilution or 20 μg/ml; 1h at room temperature). Peroxidase blocking solution (Dako Cytomation) was applied to the slides for 10 min at room temperature to inactivate endogenous peroxidase. The primary antibody was detected by use of a commercial kit (Dako REAL^{TM} Detection System, Dako Cytomation), according to the manufacturer's instructions. Finally, a 3-amino-9ethylcarbazole (AEC) chromogen (Dako Cytomation) was applied to 'visualize' the reaction and the slides

were counterstained with hemalum and covered using KP-Tape (Klinipath, Duiven, The Netherlands). IHC for bacterial pellets, normal and lymphoma tissues was performed on arrays, while IHC of pelleted cultured cells was performed on whole sections. Additionally, an antibody dilution series was tested with formalin-fixed paraffin wax-embedded bacterial pellets to define the antibody titre (Keller *et al.*, 2007b). Labelling of normal tissues was scored according to intensity as (-) negative; (+/-) faint; (+) weak; (++) moderate; and (+++) strong. Labelling of lymphoma tissues was scored by estimating the percentage of labelled cells. Prior to this evaluation, cores were checked to ensure that they were representative of the tumour by comparing B-cell and T-cell immunolabelling of consecutive sections with the results of immunophenotyping of the whole sections, and by comparing cellular morphology. Non-representative cores and cores containing prominent necrosis or autolytic change were excluded. Survivin expression was evaluated only in cases where at least one complete representative core was available.

Preincubation of Antibodies

The recombinant GST-survivin fusion protein and the GST protein alone expressed in bacterial cells were purified using gluthatione 4B sepharose beads (Amersham Biosciences), according to the manufacturer's instructions and with minor modifications. Briefly, induced *E. coli* cultures (500 ml) were centrifuged and resuspended in lysis buffer (50 mM Tris-HCl, [pH 7.5], 1 % Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 10 µg Pepstatin A/ml) and lyzed by sonication. 625 µl sepharose beads were added to the supernatant and incubated for 1 h at 4 °C with mixing by inversion. After washing, the beads were

resuspended in 2.1 ml lysis buffer and 2.9 ml 84% glycerol (50% final concentration) and separated into aliquots of 500 μl and stored at -20°C. The purity of the protein and its concentration were determined using SDS-PAGE gels stained with Coomassie blue.

Primary antibodies for survivin were diluted in commercial diluent (Dako REAL™) as described above to final concentrations of 1.6 μg/ml (pSurv) and 20 μg/ml (mSurv), respectively. Diluted primary antibodies and GST fusion protein-bound sepharose beads were incubated overnight at 4 °C, at a 10:1 antigen-antibody ratio, and mixed by inversion. Following centrifugation of the bead solution, the preincubated antibody sample supernatant was stored at 4°C and used for IHC instead of the primary antibody where appropriate.

Lymphoma Classification

Lymphomas included in this study were classified using the original tissue sections and according to the revised Kiel classification adapted for the dog as previously described (Fournel-Fleury *et al.*, 2002; Ponce *et al.*, 2004) and by the Revised European American Lymphoma – World Health Organization (REAL-WHO) classification (Valli *et al.*, 2002). The cases were immunophenotyped using sections from the original blocks (Keller *et al.*, 2007a).

Results

Cross-Reactivity of Antibodies with Recombinant Canine Survivin

To establish a reliable method for the immunohistochemical detection of canine survivin, the cross-reactivity of the two anti-human primary antibodies was determined. For this purpose, the entire coding sequence for survivin was cloned from MDCK cells.

Sequencing revealed 100% homology with the previously reported canine survivin reference sequence (GenBank Accession number NM_001003019). In western blotting with lysates of bacteria expressing canine recombinant proteins, both antibodies recognized a band of the expected molecular weight (43 kDa) corresponding to the GST-survivin fusion protein, while the reagents did not label GST alone (Figs. 1A – C). Both antibodies labelled formalin-fixed and paraffin wax-embedded bacteria expressing GST-survivin, but not bacteria expressing GST alone or other GST-tagged proteins including canine XIAP, cIAP1, cIAP2, Smac/DIABLO, or Omi/HtrA2, or normal *E. coli* (Figs. 1D and E), thus confirming the results of immunoblotting in a test system similar to the final application of this study. The antibody titres, (i.e. the inverse of the highest antibody dilutions retaining a clear-cut difference in labelling between bacteria expressing the survivin fusion protein and bacteria expressing GST alone) were 2,500 for pSurv and 40,000 for mSurv.

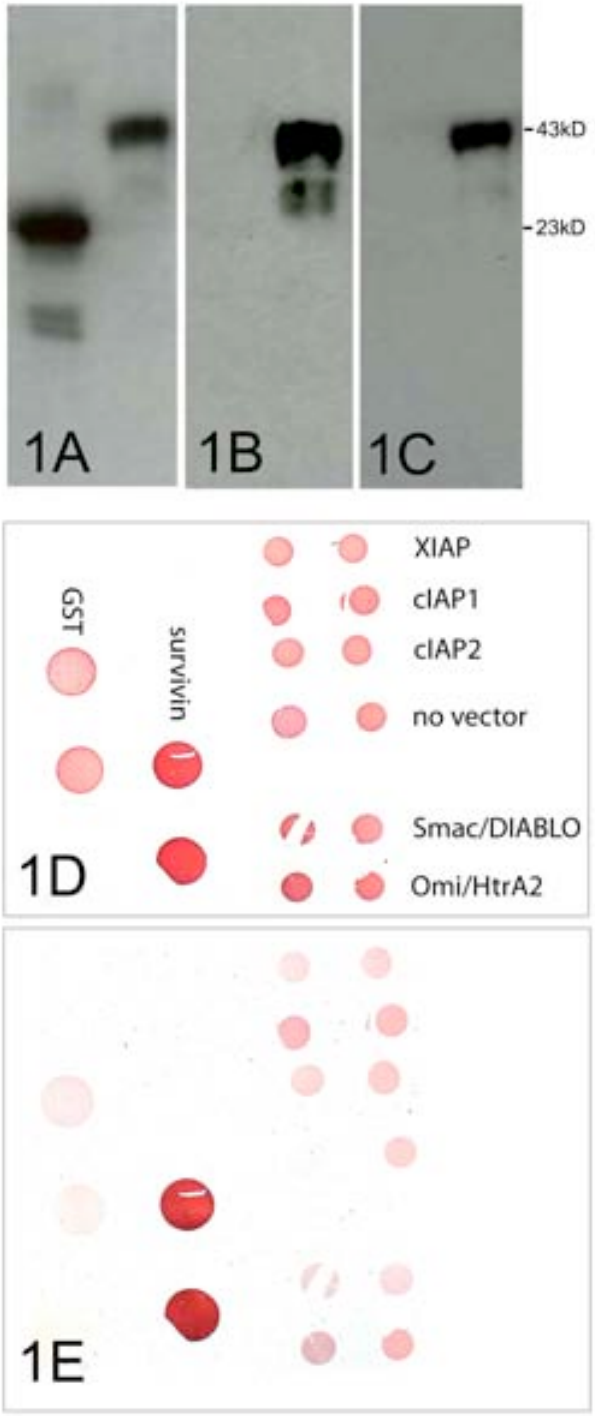

Fig. 1. Cross-reactivity and specificity of commercially available anti-human survivin antibodies against canine survivin. (A) Western blot detection of glutathione-S-transferase (GST, lane 1) and GST-survivin (lane 2) with anti-GST antibody. (B) GST and GST-survivin screened with pSurv. (C) GST and GST-survivin incubated with mSurv. (D) Labelling of an array containing formalin-fixed, paraffin wax-embedded bacteria expressing GST or GST-fusion proteins as indicated, or normal *E. coli* ('no vector'), each reaction in duplicate, with pSurv. (E) Labelling of the same bacteria with mSurv. XIAP, X-linked inhibitor of apoptosis protein; cIAP1, cellular inhibitor of apoptosis protein 1; cIAP2, cellular inhibitor of apoptosis protein 2; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI; Omi/HtrA2, Omi protease/high temperature requirement protein 2.

Immunohistochemical Detection of Survivin in Cultured Canine Keratinocytes

After demonstrating cross-reactivity of the antibodies with the canine protein, immunohistochemical detection protocols for survivin were established with cultured canine cells. Both primary antibodies labelled formalin-fixed and paraffin wax-embedded cultured non-neoplastic (Fig. 2) and neoplastic (squamous cell carcinoma) keratinocytes (data not shown). The labelling pattern was identical with both cell types and for each antibody and consisted of a predominantly multifocal cytoplasmic, finely granular signal visible in most of the cells, and a diffuse nuclear finely granular labelling present in approximately 50% of the cells. Use of a polyclonal antibody potentially risks non-specific labelling due to non-specific reactivity with unrelated antigens. To exclude this possibility, pSurv was preincubated with an excess of canine GST-survivin prior to incubation with consecutive sections of the cells. Preincubation resulted in total loss of signal, suggesting that pSurv labelling is specific (Fig. 2). The same preincubation procedure was performed with mSurv with similar results. Preincubation of pSurv with GST alone lead only to a slight attenuation of the signal (data not shown).

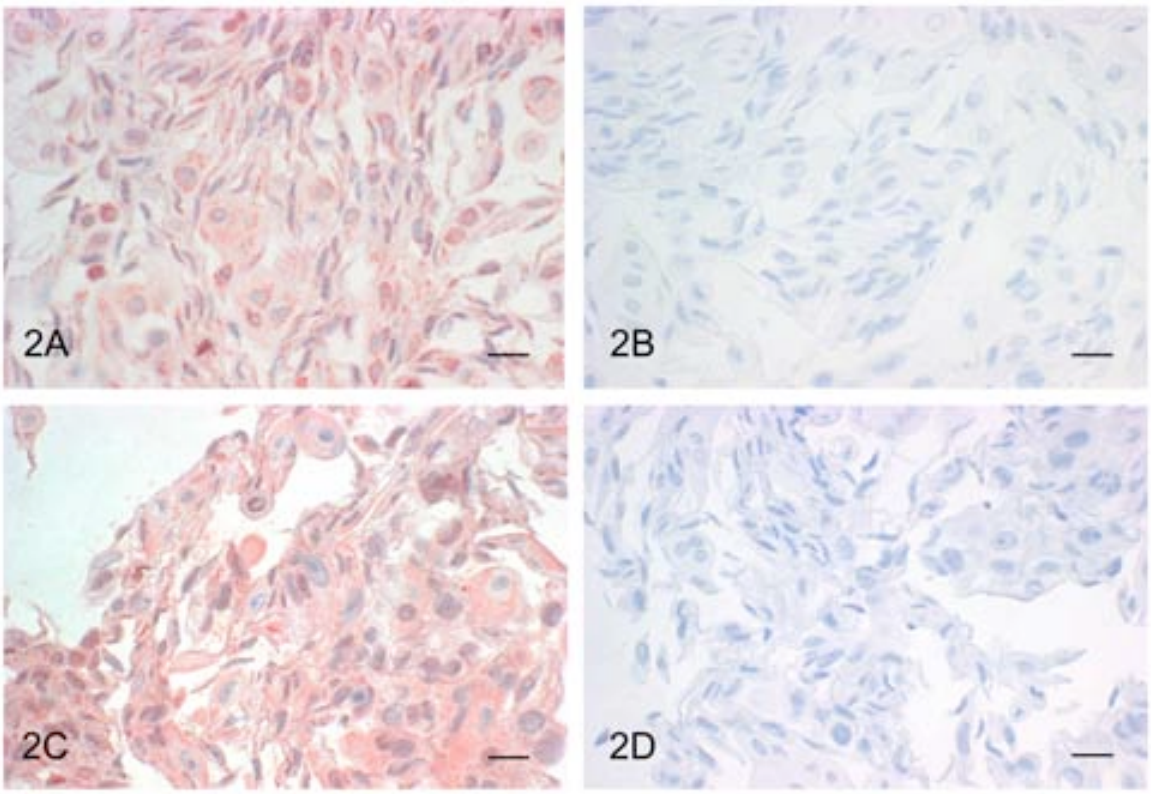

Fig. 2. Immunohistochemical labelling of cultured normal canine keratinocytes. pSurv and mSurv elicit a similar signal that is completely abrogated after preincubation with recombinant GST-canine survivin. Labelling of cells with pSurv (A – B) and mSurv (C – D) without (A and C) and after (B and D) preincubation with GST-survivin. Bar, 20 μm.

Survivin Expression in Normal Tissues

The results of this investigation are summarized in Table 1 with representative images displayed in Fig. 3. In both fetal and adult cells there was a predominant cytoplasmic expression of survivin. Most positive cell types were labelled by both primary antibodies with the exception of neurons and primary spermatocytes in adult tissues, which were labelled only by pSurv. Biliary ductal cells in adults, which were labelled with pSurv, were not available for labelling with mSurv because these structures were not represented in the section. No tissue was labelled exclusively by the monoclonal

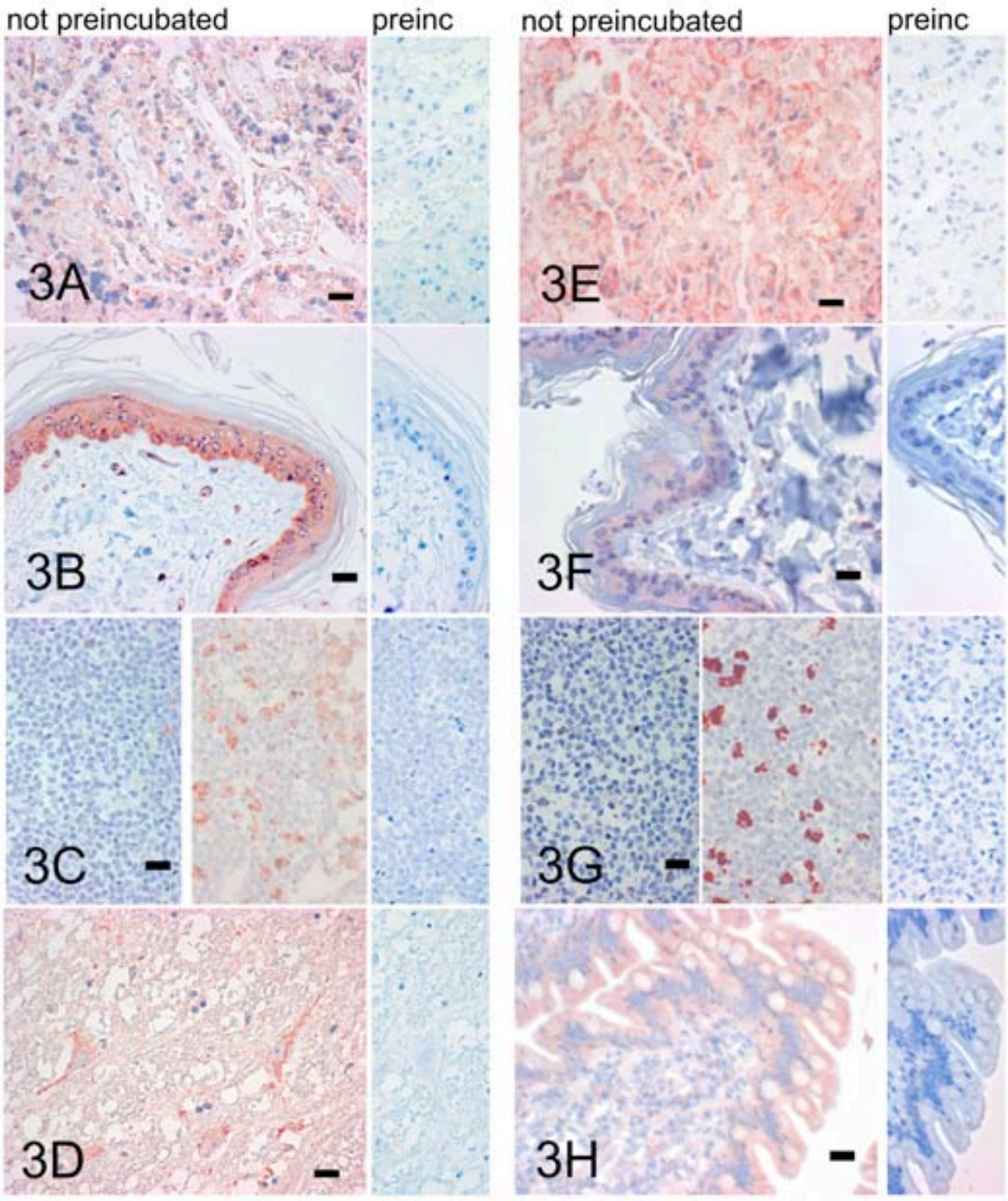
not preincubated
preinc
not preincubated
preinc
3A
3E
3B
3F
3C
3G
3D
3H

Fig. 3. Immunohistochemical labelling of normal adult dog tissues. (A – D) tissues labelled with pSurv, (E – H) tissues labelled with mSurv; not preincubated, antibody not preincubated with recombinant canine survivin; preinc, antibody preincubated with recombinant canine survivin. (A) Placenta with granular cytoplasmic labelling of trophoblast and decidua cells. (B) Skin with diffuse and granular cytoplasmic labelling of nucleated squamous epithelial cells. The stratum corneum is unlabelled and the capillary endothelia are labelled. (C) Lymph node tissue with predominantly unlabelled lymphocytes (left) and with labelled macrophages (middle). (D) Brain tissue with granular cytoplasmic labelling of neurons. (E) Placenta with diffuse cytoplasmic labelling of trophoblast and decidua cells. (F) Skin with diffuse faint cytoplasmic labelling of nucleated squamous epithelial cells and unlabelled stratum corneum. (G) Lymph node tissue with predominantly unlabelled lymphocytes (left) and with labelled macrophages (middle). (H) Intestine with diffuse cytoplasmic labelling of villus enterocytes and of scattered cells in the lamina propria. In all sections with preincubated antibody the signal is completely abrogated. Bar, 20 μm.

Table 1

Immunohistochemical survivin detection in normal fetal and adult canine tissues

		Immunohistochemical labeling result with indicated antibody			
Organ/tissue	Cell type	pSurv	pSurv preinc	mSurv	mSurv preinc
Intestine (F)	Enterocytes (villi & crypts)	++	-	+	-
	Lamina propria cells	+	-	+	-
	Myocytes (Tunica muscularis)	+	-	+	-
	Brunner's glandular cells	-	-	-	-
Pancreas (F)	Exocrine glandular cells	++	-	+	-
	Exocrine ductal cells	n.a.	n.a.	n.a.	n.a.
Brain (F)	Neuronal cells	-	-	-	-
	Glial cells	++	-	+	-
	Ependymal cells	+	-	+	-
Lung (F)	Bronchiolar epithelial cells	++	-	+	-
	Alveolar lining cells	++	-	+	-
	Macrophages	++	-	+	-
Liver (F)	Hepatocytes	++	-	+	-
	Biliary epithelial cells	n.a.	n.a.	n.a.	n.a.
Kidney (F)	Tubular cells	++	-	++	-
	Glomerular cells	-	-	-	-
Placenta (F)	Trophoblasts	+	-	+	-
Heart (F)	Myocytes	+	-	+	-
Skeletal muscle (F)	Myocytes	+	-	+	-
Spleen (A)	Lymphocytes	-	-	-	-
	Plasma cells	+	-	+	-
	Macrophages	+	-	+	-
Intestine (A)	Enterocytes (villi & crypts)	+	-	+	-
	Lamina propria cells	+	-	+	-
	Myocytes (Tunica muscularis)	n.a.	n.a.	n.a.	n.a.
	Brunner's glandular cells	-	-	-	-
Pancreas (A)	Exocrine glandular cells	++	-	+	-
	Exocrine ductal cells	-	-	-	-
Brain (A)	Neurons	++	-	-	-
	Glial cells	-	-	-	-
	Ependymal cells	n.a.	n.a.	n.a.	n.a.
Lung (A)	Bronchiolar epithelial cells	-	-	-	-
	Alveolar lining cells	-	-	-	-
	Macrophages	-	-	-	-
Liver (A)	Hepatocytes	++	+/-	+	-
	Biliary epithelial cells	++	-	n.a.	n.a.
Kidney (A)	Tubular cells	++	+/-	+	-
	Glomerular cells	-	-	-	-
Placenta (A)	Decidual cells	+	-	+	-
Heart (A)	Myocytes	+	-	+	-
Skeletal muscle (A)	Myocytes	+	-	+	-
Testis (A)	Leydig cells	++	-	+	-
	Primary spermatocytes	++	-	-	-
	Spermatids	-	-	-	-
Skin (A)	Squamous epithelial cells	++	-	+	-
	Hair follicle epithelial cells	+	-	+	-
	Adnexal glandular cells	+++	+/-	++	+/-
Lymph node (A)	Lymphocytes	-	-	-	-
	Plasma cells	++	-	++	-
	Macrophages	++	-	++	+/-

(F), fetal.; (A), adult; pSurv = polyclonal rabbit anti-survivin antibody (Novus Biologicals); mSurv = mouse monoclonal anti-survivin antibody (Santa Cruz Biotechnology); preinc = indicated antibody preincubated with recombinant canine survivin; labelling intensity scoring: -, negative; +/- faint; +, weak; ++, moderate; and +++, strong; n.a. = not available; occasional, inconsistently observed labelling of vascular endothelial cells in several organs was not indicated.

antibody. All positive cell types in fetal tissues were labelled by both antibodies. In general, labelling with mSurv was less intense than with pSurv, except for placental tissue, which was more intensely labelled by the monoclonal antibody. Moreover, labelling with pSurv was generally diffusely homogeneous with superimposed fine granules, while most labelling with mSurv was diffusely homogeneous and labelled granules were rare. Preincubation of the primary antibodies with an excess of GST-survivin resulted in complete loss of the signal in most tissues, supporting the notion that the labelling was specific for survivin. In hepatocytes, renal tubular cells and cells of the cutaneous adnexal glands, weak residual labelling persisted after preincubation of pSurv antibody either in all cores (liver, skin) or in a portion of the cores examined (kidney). The same phenomenon was observed with mSurv for cells of the cutaneous adnexal glands and in macrophages in lymph nodes. This residual reaction may have been due to the expression of unrelated cross-reacting antigens, incomplete depletion of specific antibodies during the preincubation step or the presence of antibodies not specific for survivin.

In all lymphoid tissues included in the array, the majority of lymphocytes were unlabelled, while other cells, tentatively identified as plasma cells and cells of the reticuloendothelial system, showed cytoplasmic labelling. This was confirmed by labelling whole sections of two non-neoplastic canine lymph nodes. In addition, examination of the whole sections revealed very faint nuclear staining of lymphocytes in the centre of some follicle, a finding that was not noted in the arrays due to the limited amount of tissue captured using this technique.

Survivin Expression in Canine Lymphoma

Representative cores containing sufficient amounts of tumour tissue were available for 83 of 95 tumours in the arrays labelled with pSurv and 82 of 95 tumours labelled with mSurv (Table 2). In this analysis the most frequent tumour subtypes for each immunophenotype were reported separately and infrequent subtypes and cases that could not be classified were grouped together (other subtypes/not classifiable). Examples of signals detected with the two antibodies are shown in Fig. 4. pSurv labelled a high proportion of neoplastic cells in all tumours. The pattern of pSurv labelling was similar to that described for normal non-lymphoid tissues. In general, the percentage of labelled tumour cells and the signal intensity were lower with mSurv. Nevertheless, mSurv labelled more than 5% of cells in 66 of 82 tumours and more than 40% of cells in 51 of 82 tumours. The labelling pattern for mSurv was predominantly cytoplasmic and diffuse. In all lymphoma tissues, labelling by both antibodies was completely lost following preincubation with survivin-GST.

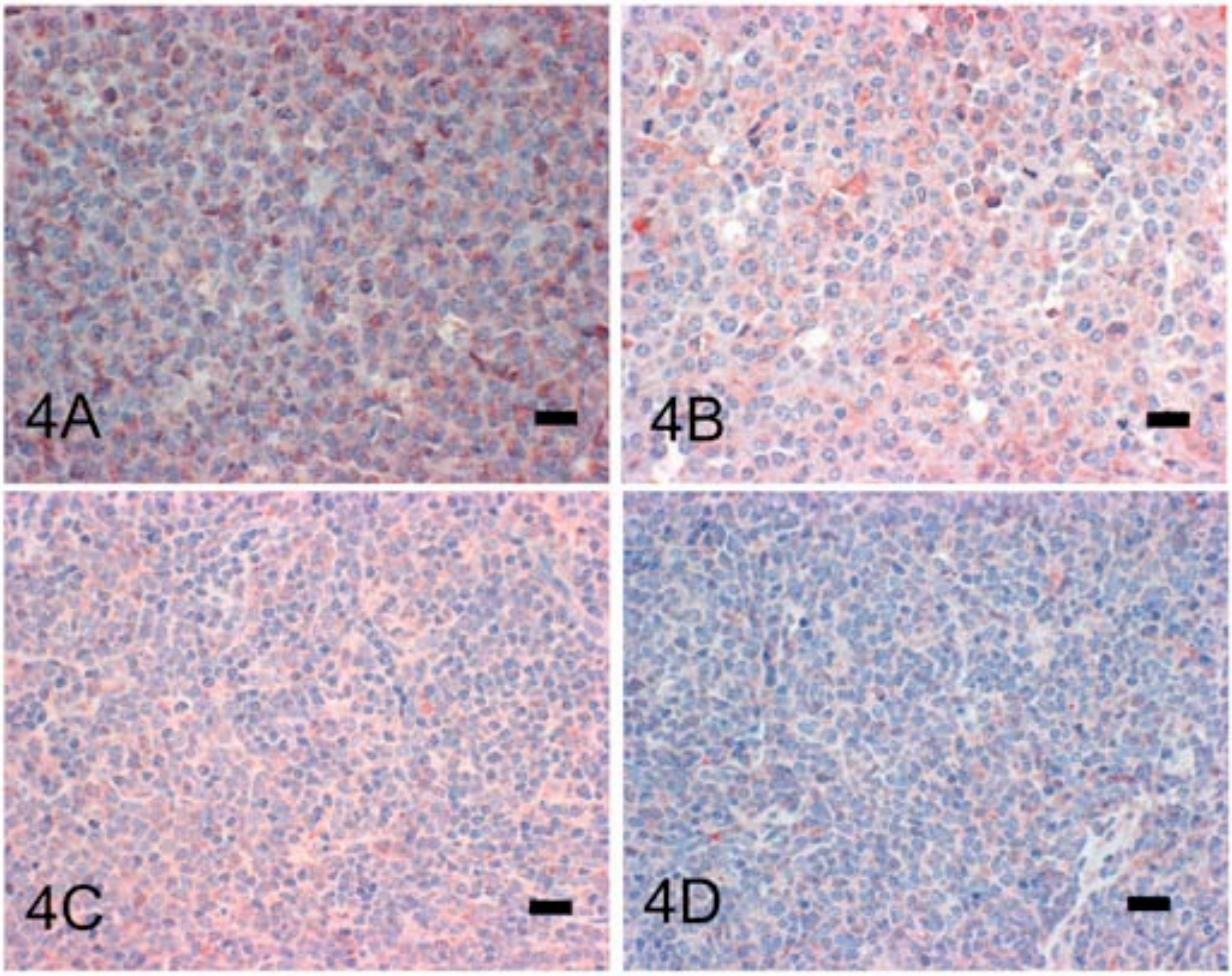

Fig. 4. Immunohistochemical labelling of canine lymphoma tissues. (A – B) Centroblastic monomorphic lymphoma (Kiel classification) / diffuse large B-cell lymphoma (REAL-WHO classification). (C – D) Centroblastic polymorphic lymphoma (Kiel classification) / diffuse large B-cell lymphoma (REAL-WHO classification). Labelling with pSurv (A and C) or mSurv (B and D). Bar, 20 μm.

Table 2

Immunohistochemical detection of survivin in canine lymphomas

Lymphoma subtype according to indicated classification scheme		No. of tumours with indicated percentage of positive cells detected with indicated antibody								
Revised Kiel	REAL-WHO	Total no. of cases	pSurv				mSurv			
			0-5%	6-39%	40-70%	>70%	0-5%	6-39%	40-70%	>70%
B-Immunophenotype (n=45)										
centroblastic monomorphic	diffuse large B-cell	7	0	0	1	6	1	1	1	4
centroblastic polymorphic	diffuse large B-cell	17	0	0	1	16	2	1	6	8
marginal zone	marginal zone	11	0	0	1	10	2	4	2	3
other subtypes / not classifiable		10	0	0	0	10	3	1	3	3
T-Immunophenotype (n=28)										
pleomorphic (various sizes)	peripheral T-cell not otherwise specified	14	0	0	0	14	2	1	4	7
large granular	blastic Natural Killer-cell	5*	0	0	0	5	1	3	0	0
other subtypes / not classifiable		9	0	0	0	9	1	2	1	5
Double Negative-Immunophenotype (n=10)										
high-grade, medium sized		4	0	0	0	4	1	0	1	2
other subtypes / not classifiable		6	0	0	1	5	3	2	0	1
TOTAL		83*	0	0	4	79	16	15	18	33

* in one case tissue not available for analysis with mSurv; pSurv = polyclonal rabbit anti-survivin antibody (Novus Biologicals); mSurv = mouse monoclonal anti-survivin antibody (Santa Cruz Biotechnology).

Discussion

The present study has examined immunohistochemically the expression of survivin in normal canine tissues and samples of lymphoma. Reports of the expression of this protein in normal adult tissue vary (Andersen *et al.*, 2007; Lechler *et al.*, 2007; Altieri, 2008). The results of the present study indicate that cytoplasmic expression of survivin is widespread in normal fetal and adult canine tissues and that it is nearly ubiquitous across canine lymphoma subtypes. In tumour cells, cytoplasmic expression of survivin has been suggested to contribute to tumorigenesis through its anti-apoptotic function (Stauber *et al.*, 2007).

The analysis of canine survivin expression was optimized by screening two commercially available human anti-survivin antibodies against recombinant canine survivin by a variety of methodologies. IHC of paraffin wax-embedded bacteria allows exclusion of non-specific reactions with related proteins once the corresponding cDNAs are available. The majority of immunohistochemical studies on human tissues report use of polyclonal antibodies for detection of survivin, and these are considered to be more sensitive than monoclonal antibodies (Grossman *et al.*, 1999; Ding *et al.*, 2006; Vischioni *et al.*, 2007). The polyclonal antibody used in the present study has been used previously to label survivin in human specimens (Martinez *et al.*, 2004; Schlette *et al.*, 2004; Ding *et al.*, 2006; Piras *et al.*, 2007; Vischioni *et al.*, 2007) as well as in canine mastocytoma, urinary bladder transitional cell carcinoma and lymphoma (Scase *et al.*, 2006; Rebhun *et al.*, 2008; Velando Rankin *et al.*, 2008). In two of the latter studies (Scase *et al.*, 2006; Rebhun *et al.*, 2008), antibody specificity was confirmed by western blot analysis. However, the quality of polyclonal antibodies may vary significantly between different batches and therefore it is advisable to test such antibodies whenever

possible before use (Keller *et al.*, 2007b). While most previous studies were based on the use of a single antibody, the present work confirmed labelling results obtained with the polyclonal antibody by application of the monoclonal antibody mSurv. In general, there was high overall agreement in labeling by these two reagents with differences limited to a weaker signal intensity with mSurv. Since the range of organs showing signals and the reaction pattern were similar with both antibodies, the differences observed likely reflect the higher sensitivity of the polyclonal reagent. Similar findings have recently emerged in a detailed comparison of the labeling of human non-small cell lung carcinomas by the same two antibodies (Vischioni *et al.*, 2007). In that study both antibodies recognized both cytoplasmic and nuclear full length survivin as well as the alternatively spliced forms ΔEx3 and 2B ectopically expressed in a Human Breast adenocarcinoma cell line (MCF-7) cells. A possible explanation for rare discrepancies in labeling of certain cell types observed in the present study, including neurons in the brain and primary spermatocytes, may reside in the differential recognition of further alternatively spliced survivin isoforms or could derive from non-specific reactions. The latter were at least partially excluded by preincubation of the primary antibody with recombinant canine survivin as a negative control. Overall, it would appear that pSurv is a reliable and sensitive tool for the detection of survivin in dog tissues.

These antibodies identified widespread and predominantly cytoplasmic expression of survivin in normal canine fetal tissues as has been described for man and mouse (Ambrosini *et al.*, 1997; Adida *et al.*, 1998). In contrast, it appears that survivin expression is more widespread in normal adult canine tissues than previously reported. The finding of survivin expression in epithelia of the placenta, gastrointestinal tract and skin are in agreement with multiple immunohistochemical studies of adult human and

rodent tissues (Chiodino *et al.*, 1999; Gianani *et al.*, 2001; Ding *et al.*, 2006; Lechler *et al.*, 2007; Piras *et al.*, 2007) and with expression data available from a publicly accessible repository (http://www.proteinatlas.org/). Positive expression in several further organs including testis, kidney, liver and brain (Kobayashi *et al.*, 1999; Altura *et al.*, 2003; Moon and Tarnawski, 2003; Lechler *et al.*, 2007) is supported by at least one previous study with human tissues and by data from the protein atlas. One study in particular has demonstrated, with different methods, high survivin levels in the adult renal proximal tubules (Lechler *et al.*, 2007). Failure to report positivity of such organs in several other reports may relate from the fact that normal tissues were either not mentioned or not investigated in a cancer study, or from the use of different antibodies or IHC protocols. Another reason may relate to individual variations in the expression of this protein. For instance, in a recent study, cytoplasmic survivin labelling was detected in the epithelium in only 17 of 46 normal canine urinary bladders (Velando Rankin *et al.*, 2008). Several additional tissues with reported positive cell types including oesophageal epithelium and bone marrow (Fukuda and Pelus, 2006) were not included in the present study but these tissues from dogs might also express survivin. In the present study, a more extensive identification of cell types was not possible in the tissue microarrays due to the limited amount of tissue examined and the limited number of tissues included. Furthermore, the small number of individuals studied precludes assessment of individual variations. However, the data point to widespread expression of survivin in normal adult canine tissues. A more detailed study is warranted, particularly considering the fact that therapies targeting this molecule are currently under investigation in man and are not devoid of side effects (Altieri, 2008).

Previous reports indicate that survivin is frequently expressed in most cancer types (Andersen *et al.*, 2007; Altieri, 2008). The findings of the present study suggest that most, if not all, canine lymphomas express survivin and that this molecule might have a role in the genesis or maintenance of these tumours. While pSurv consistently labelled most tumour cells in the majority of the cases analyzed, mSurv labelled fewer cells in a substantial number of tumours, suggesting differences in the level of expression between lymphomas. In a recent report, a negative prognostic significance was ascribed to high levels of survivin expression assessed immunohistochemically in samples of canine B-cell lymphoma (Rebhun *et al.*, 2008). In that study, survivin was detected in all tumour samples investigated, which is in accordance with our data based on a larger sample size. Those authors did not report the relative extent of nuclear and cytoplasmic labelling, while in the present investigation cytoplasmic survivin appeared to be predominant. In man, survivin is immunohistochemically detected in high grade rather than in low grade lymphomas, which are considered negative (Ambrosini *et al.*, 1997; Tracey *et al.*, 2005). In diffuse large B-cell lymphoma, survivin expression, detected by IHC, was reported in 43 – 80% of cases either in the nucleus (Watanuki-Miyauchi *et al.*, 2005; Liu *et al.*, 2007), in the cytoplasm (Adida *et al.*, 2000; Ambrosini *et al.*, 1997) or without mention of subcellular localization (Tracey *et al.*, 2005). In mantle cell lymphoma, another subtype of B-cell lymphoma, survivin expression was reported to be rare (Tracey *et al.*, 2005), while another report described mean expression of 10 – 30% of tumour cells with dominant nuclear labelling (Martinez *et al.*, 2004). Importantly, this latter study, in which pSurv was used, confirmed subcellular localization by western blotting of lysates of tumour cell lines and of tumour cells isolated from patients and subjected to subcellular fractionation. Another group, using the same antibody,

identified cytoplasmic survivin expression in 55% of anaplastic large-cell lymphomas (a subtype of lymphoma mostly comprising T-cells) and confirmed the subcellular localization in cell lines (Schlette *et al.*, 2004). In contrast to these studies in man, both the present investigation and a previous study point to widespread expression of survivin across most subtypes of canine lymphoma (Rebhun *et al.*, 2008).

In conclusion, by application of a combination of two different antibodies against survivin and screening of arrays of normal tissue, the present study has shown widespread cytoplasmic survivin expression in normal adult canine tissues. These observations should be taken into account when developing therapies targeting this molecule for the management of neoplastic disease.

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Conflict of Interest Statement

The authors have no conflict of interest.

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